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(54) Title: **DECONDENSATION OF DNA**

(57) Abstract: Decondensation of DNA is accomplished by treating DNA-containing cells with a thiol and, simultaneously or subsequently, with anionic surfactant wherein the cells are exposed to the thiol for less than 20 minutes. The cells are preferably sperm cells and the method is particularly useful in livestock sperm such as bovine sperm. Sperm cells may be subjected to fluorescent in situ hybridisation after decondensation. The invention also provides kits comprising thiol and anionic surfactant and treatment instructions. The methods and kits are useful in assessing the efficiency of sperm sorting procedures.

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DECONDENSATION OF DNA

5 This invention relates to methods for the decondensation of DNA, especially sperm DNA, and to the use of such decondensation methods in processes of validation of sperm separation procedures, especially bovine and other livestock sperm separation procedures.

10 Sex determination of progeny before fertilisation is now regarded as an important and realistic development, especially in the cattle industry. It is a development that could improve the economics and management of both dairy and beef breeder operations (Amann RP, 1999). The Beltsville Sperm Sexing Technology is the only method at  
15 present that has proven effective in sexing viable sperm (Johnson LA, 1995). This process, which uses flow cytometry and sperm sorting, was started in the late 1980s (Johnson LA et al., 1989) but has improved considerably since (Rens et al., 1996; Rens et al., 1998; Johnson LA,  
20 Welch GR and Rens W, 1999; Johnson LA and Welch GR, 1999; Rens et al., 1999). Improvements were also directed at alternative procedures for artificial insemination (Johnson LA, 1995; Catt et al., 1996; Rath et al., 1997; Seidel et al. 1997; Long et al., 1998).

25 However, alternative methods of sorting X- and Y-bearing sperm are still being researched (Blecher, 1999; Hendriksen PJM, 1999; Van Munster, 1999) to develop methods to sex large numbers of sperm inexpensively. The flow cytometric sorting method is limited in speed and output  
30 because it is based on the analysis and sorting of one sperm at a time.

All of these endeavours would benefit from the availability of a quantitative method for validating the efficiency of X and Y sperm separation. Checking the sex  
35 of the embryo or offspring is laborious and delays the development of the system. Flow cytometric analysis is also used to assess purities (Welch GR and Johnson LA,

1999). However, a flow cytometer and, more importantly, an experienced biologist are required to obtain reliable results. Application of the PCR method to single sorted X- and Y-sperm has been performed successfully, but is time consuming (Welch et al., 1995).

Another possibility is to use fluorescence in situ hybridisation (FISH) of sexed sperm. For this, separate probes for both X- and Y-bearing sperm are needed to avoid false-negative results.

In order to carry out a FISH protocol effectively, decondensation of the sperm DNA is necessary. Various methods for such decondensation have been described. Most published protocols for sperm decondensation use a combination of DTT (dithiothreitol) and LIS (lithiumdiiodosalicylate) mainly for human sperm (Martin and Ko, 1995), or DTT alone for porcine spermatozoa (Kawarasaki et al, 1996). However, we have found that when bovine sperm are exposed to these reagents, adequate sperm enlargement is not observed. DTT in combination with heparin does enlarge bovine sperm heads but this is due to a removal of the acrosome after which DNA was released. This process does not provide clear FISH signals.

The method reported by Hassanane et al., 1999 uses DTT and papain for bovine sperm and results in the need to select an area on the slide with optimum sperm decondensation.

A further method is reported by Rodriguez et al., 1985. This method uses DTT, sodium lauryl sulphate and sodium borate. This method was devised by the authors in order to measure resistance of ovine sperm to the decondensation process, stable sperm being those which did not exhibit decondensation. The authors were interested in correlation of decondensation with fertility. They were not concerned with identification of X- and Y- bearing sperm.

Neither of these methods is suitable for the application of FISH to sperm DNA, in particular bovine

sperm DNA. Neither method results in decondensation to a degree which allows consistent and easy to detect FISH signals. We find that the Rodriguez method has the disadvantage that it tends to disintegrate DNA. The  
5 Hassanane method has the disadvantage that it gives insufficient and inconsistent decondensation.

Therefore it would be desirable to provide a method of sperm decondensation which does not disintegrate DNA, is quick and reproducible and leads to a level of  
10 decondensation similar for every sperm in the sample. In particular, it would be desirable to provide a decondensation protocol which leads to the achievement of clear hybridisation signals in a FISH protocol.

According to the invention we provide a method of  
15 decondensation of DNA, especially sperm DNA, comprising treating cells containing DNA, especially sperm cells, with a thiol and, simultaneously or subsequently, anionic surfactant, wherein the time of exposure to the thiol is not more than 20 minutes.

20 The method is especially applicable to sperm cells, in particular livestock sperm such as bovine, porcine, ovine or equine sperm. We find that the method of the invention is particularly suitable for application to bovine sperm, especially in systems where X and Y bearing sperm are to be  
25 detected by FISH. This method is particularly useful when chromosome paints are used to distinguish X bearing sperm from Y bearing sperm. These are highly target specific and result in a clear signal, but the protocols described previously are inappropriate for, in particular, bovine  
30 sperm labelling.

The technique does not disintegrate the DNA, is quick and reproducible and leads to a level of decondensation similar for every sperm in the sample. This avoids the need to search for an area on the slide with optimum sperm  
35 decondensation, as in Hassanane et al 1999. This robust and reproducible procedure is simple to use in demonstrating effectiveness of any putative sexing

technique. Thus the invention will assist the development of alternative, faster X-Y sperm sorting techniques by the introduction of this rapid validation method.

The invention also provides a method of distinguishing  
5 X bearing sperm from Y bearing sperm by fluorescence in situ hybridisation (FISH), the method comprising decondensation of the sperm DNA as described above.

The invention also provides a kit for use in decondensation of DNA comprising (A) a thiol and (B)  
10 anionic surfactant and (C) instructions to treat cells containing DNA with the thiol and anionic surfactant such that the time of exposure to the thiol is not more than 20 minutes.

The invention also provides a kit for use in decondensation of DNA comprising (A) a solution of thiol at  
15 a concentration of at least 50mM and (B) a solution of anionic surfactant.

The DNA decondensation method of the invention may be applied to DNA from any cell type, but is particularly  
20 useful for decondensation of sperm DNA. Thus the invention will be discussed below in the context of decondensation of sperm DNA.

The sperm are preferably treated with the thiol prior to treatment with the anionic surfactant. The thiol is  
25 preferably dithiothreitol (DTT). This is believed to reduce disulphide bonds. The concentration of thiol to which the sperm are exposed is often at least 25 mM, preferably at least 75 mM, in particular from 100 to 200 mM. Generally it is not more than 1 M or 500 mM. A  
30 concentration of about 125 mM has been found to be particularly useful.

The thiol is often supplied as an aqueous solution, in which the concentration of thiol can be higher than the concentration to which the sperm are to be exposed. For  
35 instance, the concentration of the solution of thiol as supplied (for the method of the invention or as used in the kit of the invention) can be at least 50mM, preferably at

least 150mM, often at least 200mM. A concentration of about 250mM has been found to be particularly useful. Suitable upper limits on the concentration as supplied are 1M or 500mM.

5       The sperm are exposed to the thiol for not more than 20 minutes (in contrast to the Rodriguez method in which exposure is for up to 45 minutes). Preferably exposure is for not more than 10 minutes, more preferably not more than 5 minutes, in particular not more than 3 minutes. Exposure  
10       times of about 2.5 minutes have been found to be particularly beneficial. Exposure is generally for at least 30 seconds, preferably at least 1 minute.

      We find that the combination of a higher concentration of thiol and a shorter exposure time in comparison with the  
15       Rodriguez method is beneficial, especially in treatment of bovine sperm for FISH.

      The sperm are exposed to anionic surfactant. Any suitable anionic surfactant may be used, for instance alkyl sulphates and alkyl benzene sulphonates, but alkyl  
20       sulphates are preferred, in particular dodecyl sulphates such as sodium dodecyl sulphate (SDS).

      Time of exposure to the surfactant is generally not more than 1 minute, often not more than 30 seconds, and a time of about 10 seconds has been found optimum. Exposure  
25       is generally for at least 2, preferably at least 5 seconds.

      The concentration of anionic surfactant in the treatment environment may be from 0.01 to 10% (weight per volume), preferably 0.1 to 3%, more preferably 0.2 to 1%,  
30       and a concentration of around 0.5% has been found to be optimum.

      The concentration of surfactant in the solution applied to the treated sperm (and in the kit of the invention) is often around twice the concentration to which  
35       the sperm are exposed. It may be from 0.02 to 20 wt%, preferably 0.2 to 6%, more preferably 0.5 to 2%, eg about 1%).

It is also highly desirable to expose the sperm to a borate salt. This aids in keeping sperm intact, avoiding the sperm becoming speckled and balloon shaped. Preferably exposure to the borate salt is simultaneous with exposure to the anionic surfactant. More preferably exposure to borate salt occurs only when the sperm are exposed to anionic surfactant.

The borate salt is preferably sodium ditetraborate.

Suitable concentrations of borate salt are from 0.05 to 10% (weight by volume), preferably 0.1 to 5 %, more preferably 0.2 to 2%. A concentration of about 0.9% has been found to be optimum.

The concentration of borate salt in a solution applied to the sperm (and in the kit of the invention) is often about twice the above concentrations. That is, it can be from 0.1 to 20 wt%, more preferably 0.2 to 10wt%, more preferably 0.4 to 4 wt%. A concentration of around 1.9% has been found to be optimum.

Preferred times of exposure to the borate salt are the same as for the anionic surfactant.

Preferably exposure to the thiol, anionic surfactant and borate salt (if used) is at room temperature, usually from 16 to 25°C, generally 16 to 20°C.

We have found that the sperm concentration in the treated sample can be important. It is preferably from  $10^7$  to  $10^{10}$  sperm/ml, more preferably  $10^8$  to  $10^9$  sperm/ml, most preferably  $1.5$  to  $3.5 \times 10^8$  sperm/ml, and a concentration of about  $2.5 \times 10^8$  sperm/ml has been found to be optimum.

We have also found that the ratio between the sperm concentration and the concentration of treatment reagents is important and if the sperm concentration is changed then preferably the ratios remain similar, by means of dilution of the reagents.

For instance, an optimum ratio of sperm concentration to thiol concentration is  $2.5 \times 10^8:125$ , ie 2,000,000 sperm/ml:mM. Suitable ratios are from 10,000,000 to 100,000; preferably from 4,000,000 to 500,000.

Similarly, an optimum ratio of sperm concentration to anionic surfactant concentration is  $2.5 \times 10^8:0.5\%$ , namely  $5 \times 10^8$  sperm/ml:%. Preferred ranges of this ratio are from  $5 \times 10^7$  to  $5 \times 10^9$ .

5 Similarly, preferred ratios of sperm concentration to concentration of borate, if used, are from  $5 \times 10^7$  to  $5 \times 10^9$  sperm/ml:%.

After decondensation with thiol, anionic surfactant and optionally borate salt, the decondensed DNA is  
10 preferably fixed using ethanol. This can be by addition of, for instance, 70% ethanol at least 5 times, preferably at least 10 times, total volume. This is in contrast with the Rodriguez method in which fixing is with glutaraldehyde.

15 If decondensation is found to be ineffective in any particular process, the concentration of the reagents should be modified to maintain effectiveness, rather than modifying the treatment times.

Preferably the sperm are washed, generally in saline,  
20 prior to treatment with the thiol and anionic surfactant. Preferably the washed sample is centrifuged, more preferably at 800 g or less. 400 g has been found to be optimum. Centrifugation time is generally from 5 to 15 minutes, and about 10 minutes has been found to be optimum.

25 The sperm treatment is carried out at a pH from about 7 to about 10, preferably from 8 to 9.

The reagents are normally supplied in aqueous solution. Preferred concentrations of as-supplied solutions are discussed above. We find that better results  
30 are achieved if the sperm are treated in suspension, normally aqueous suspension, for instance in a test tube, rather than on a slide. The concentrations mentioned above are concentrations in the treatment suspension as a whole when suspension treatment is used (unless otherwise  
35 stated). If treatment on a slide is used, then the volume of the treatment suspension is the same as the volume of the suspension or solution of reagents applied and the



concentrations are the same as those in these suspensions or solutions.

Optionally, the sperm may be treated with pepsin subsequent to decondensation. In some cases this is beneficial when the method is part of a process in which X and Y bearing sperm are identified by means of FISH, in particular chromosome painting, in that it can give reduced background painting and better contrast.

The DNA decondensation method is particularly beneficial in a method in which X and Y bearing sperm are identified by means of FISH. Known techniques may be used. X and Y specific probes may be used although preferably chromosome paints are used. These may be produced in known manner, in particular by PCR amplification of X and Y chromosomes. Yak chromosomes have been found to be suitable.

The invention will now be illustrated with reference to the following example system. This example describes the production of chromosome paints, preparation and decondensation of sperm, subsequent pepsin treatment, application of chromosome paint to the sperm and detection.

#### Example

##### Materials and methods

##### Paint production

A primary cell culture was obtained from skin material of a male Yak (*Bos grunniens*). Cells were grown at 37°C in Dulbecco's modification of minimal essential medium (BRL) enriched with 15% fetal bovine serum (BRL), penicillin (100 units/ml), streptomycin (100 µg/ml) and glutamine (2mM). Chromosomes were isolated as described previously by Yang et al., 1995. The chromosomes were prepared for sorting by staining with 40 µg/ml Chromomycin A3 (Sigma), 2 mM MgSO4 and 2 µg/ml of Hoechst 33258 (Sigma) and incubated for at least 2h. Ten minutes before flow analysis, sodium sulphite and sodium citrate were added to a final concentration of 10mM and 25mM respectively. The stained chromosome preparations were sorted on a Facstar Plus flow sorter

(Becton Dickinson) equipped with two 5W argon ion lasers. Four hundred of chromosome X and chromosome Y were sorted directly into separate 500  $\mu$ l PCR tubes containing 30  $\mu$ l of sterile distilled water. Flow sorted chromosomes were used  
5 as templates for amplification by degenerate oligonucleotide-primed PCR (DOP-PCR) (Telenius et al., 1992) using 6-MW (5'CCG ACT CGA GNN NNN NAT GTG G3' where N= any base, 2pM). Primary DOP-PCR products were used as a source of template for the incorporation of biotin16-dUTP  
10 (Boehringer) or Cy3-dUTP (Amersham). Fifty ng each of the X and Y paint were made up to 15  $\mu$ l with hybridization buffer (50% deionised formamide, 10% dextran sulphate, 2XSSC, 0.5M phosphate buffer, pH 7.3).

#### Washing of sperm

15 Unsorted fresh sperm was supplied in a tris-based ambient temperature extender and was checked for its concentration. Flow sorted sperm was supplied in a TEST-yolk medium at a concentration of  $1.5 \times 10^6$  sperm/ml. Sperm samples were washed in 0.01 M Tris, 0.9% NaCl and spun at  
20 400g for 10 min to remove the supernatant. A final concentration of  $2.5 \times 10^8$  sperm/ml was obtained by adding 0.01 M Tris, 0.9% NaCl.

#### Decondensation of sperm

A volume of washed sperm was put in a 0.5 ml eppendorf  
25 and was frozen and thawed rapidly by immersion in liquid nitrogen. An equal volume of 0.25M DTT (dithiothreitol) in 0.01M Tris, 0.9% NaCl was added and the tube was incubated for 2.5 mins at room temperature. Subsequently, an equal volume of 1% (w/v) Sodium lauryl sulphate, 1.9% (w/v) di  
30 Sodium tetraborate was added and incubated for 10 sec after which 70% ethanol was added at 10 times the total volume of the other reagents. A droplet of 2 $\mu$ l of decondensed sperm was deposited on a slide. The slide was immediately dried at 50°C on a hot plate. The slide was dehydrated in 100%  
35 ethanol for 5 min and dried at room temperature. Sperm should be checked at this stage with a phase contrast microscope. The sperm tail should remain attached but

deformed into a curled shape (Fig. 1c). If sperm tails are straight (Fig. 1a), only slightly affected (Fig. 1b) or absent (Figs. 1d, e), this step should be carried out on a new sample, modifying the conditions. Different dilutions of the reagents should be used to obtain the correct decondensation, as this is more effective than changes in the duration of treatment.

#### Pepsin treatment (optional)

The slide was incubated in 0.01% pepsin (Sigma) in 10 mM HCL for 30 min at room temperature. Subsequently, the slide was washed 3 times in 2XSSC for 1 min each and rinsed with distilled water. The slide was then dehydrated by putting it through an ethanol series: twice 2 min 70% ethanol, twice 2 min 90% ethanol, once 5 min 100% ethanol and air dried at room temperature.

#### Hybridisation

The Yak X and Y chromosome paints were denatured for 10 min at 70 °C and chilled on ice. The slide was baked at 65°C for 30 min, incubated for 20 min in 70% formamide in 2XSSC at 80°C to denature sperm DNA and quenched in ice cold 70% ethanol for 5 min. The slide was dehydrated through a series of ethanol: twice 2 min 70% ethanol, twice 2 min 90% ethanol, once 5 min 100% ethanol and air dried at room temperature.

A 10 µl droplet of denatured chromosome paint was applied to the slide, covered with a glass cover slip and sealed with rubber solution. The slide was incubated overnight in a moist container at 37 °C.

#### Detection

After incubation, the rubber solution and cover slip were removed and the slide was washed twice for 5 min in 50% formamide in 2XSSC at 43 °C, twice for 5 min in 2XSSC at 43°C and three times for 3 min in 4XSSC containing 0.055% Tween 20 (Sigma) at 43°C. The biotin labelled X-probe was visualised with avidin-FITC (1/500 in 4XSST)/anti avidin-FITC (1/250 in 4XS ST. Vector Laboratories) solution; 200 µl of this solution was deposited on the slide and

covered with parafilm. The directly Cy3 labeled Y paint does not need signal visualization/amplification. The slide was incubated for 20 min at 37°C and washed three times for 3 min in 4XSST at 43°C. Sperm DNA was counterstained with 0.08 µg/ml 4'6-diamidino-2-phenylindole (DAPI, SIGMA) solution in 2XSSC for 2 min and the slide was mounted in Citifluor antifade AF1. Sperm were observed with a Leica DMRXA fluorescence microscope equipped with an automated filter wheel with Cy3, FITC and DAPI specific filters (LEICA Microsystems). For each experiment at least 100 sperm were counted. Images were captured using Leica QFISH with a cooled CCD camera (Photometrics Sensys) through 40X or 63X objectives.

#### Flow cytometrically sorted sperm

On two separate days bovine sperm samples were sorted using the Beltsville Sperm Sexing Technology in Beltsville, MD, USA (Johnson, 1995). Sperm were deliberately sorted with purities near 90% and 75%. The purities of the sorted samples were assessed by flow cytometric reanalysis of an aliquot of the samples. This aliquot was first sonicated to obtain sperm nuclei after which Hoechst 33342 was added to obtain uniform nuclear staining. Analysis of the aliquots produced histograms that were fitted to a double gaussian curve to determine the proportions of X- and Y-populations. This procedure is proven to be an accurate assessment of purities (Welch GR and Johnson LA, 1999). The sorted samples as well as unsorted samples (sperm concentration  $1.5 \times 10^6/\text{ml}$ ) were shipped on dry ice to Cambridge, UK. However, documentation of the purities of these samples was only provided after scoring with the developed X-Y probes to prevent prejudice.

#### Results

##### Decondensation of sperm DNA

All sperm on the slide showed the same degree of decondensation. The condition of the tail of the sperm was a measure of sperm expansion. Sperm of the type in Fig 1a exhibited weak hybridization signals. Figure 1b presents a

sperm where decondensation has just started, while Fig 1c illustrates a sperm with a curly tail. These sperm had clear hybridisation signals. Large and sometimes fragmented FISH signals were obtained with sperm where the tail was just removed (Fig 1d) or with progressively decondensed sperm (Fig 1e). It was noted, by the condition of the sperm tails, that the flow cytometrically sorted sperm were more sensitive to the decondensation reagents. This was possibly due to the necessary freezing and thawing process of the sperm samples as they were shipped on dry ice. A five to ten times dilution of the reagents appeared to be optimal for these samples.

#### X-Y Labeling of metaphase chromosomes

Fig 1f shows the results of hybridizing the X-Y probe set to a bovine metaphase and illustrates the precise specificity of the probes for their respective chromosomes.

#### X-Y labelling of non-sorted sperm

Fractions of X-chromosome bearing and Y-chromosome bearing sperm were first determined in three samples containing non-sorted fresh sperm. The percentages of X- and Y-sperm assessed with the X-Y probe Set are presented in Table 1.

#### X-Y labelling of flow cytometrically sorted sperm

Figs 1g to 1l show hybridization results on different sorted sperm samples. Three different X-sort purities are illustrated by Figs 1g-1i and three Y-sperm sorts are shown in Figs 1j to 1l.

Table 2 shows the comparison between the purity of the sorted samples assessed by reanalysis and by use of the X-Y probe set. The use of the X-Y probe for purity assessment resulted in percentages in close agreement with the reanalysis results. With the exception of sample 3 of the first day, the purity assessments were very similar. Two samples showed a percentage of sperm that were double labeled. These were sperm with weak X/Y signals. This artifact did not affect the agreement between both purity assessments.

Table 1

Sample	X%	Y%	Unlabeled %
1	50	45	5
2	47	47	6
3	44	48	8

Table 2

Sample	Reanalysis (%)		X-Y probe set (%)			
	X	Y	X	Y	unlabeled	double labeled
1, day1	8	92	3	89	8	-
2	50	50	46	50	4	-
3	87	13	76	19	5	-
4	50	50	47	47	6	-
5	91	9	85	5	10	-
6	18	82	12	82	4	2
1, day2	5	95	3	93	4	-
2	91	9	97	2	1	-
3	79	21	74	24	2	-
4	20	80	16	78	3	3

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CLAIMS

1. A method of decondensation of DNA comprising treating cells containing DNA with a thiol and, simultaneously or  
5 subsequently, treating the cells with anionic surfactant, wherein the cells are exposed to the thiol for less than 20 minutes.
2. A method according to claim 1 comprising treating the cells with the thiol and subsequently treating the cells  
10 with anionic surfactant.
3. A method according to claim 1 or claim 2 comprising treating the cells with a borate salt, preferably at the same time as the treatment with anionic surfactant, more preferably only when the cells are treated with anionic  
15 surfactant.
4. A method according to any preceding claim in which the cells are exposed to the thiol for not more than 10 minutes, preferably not more than 5 minutes, more preferably not more than 3 minutes.
- 20 5. A method according to any preceding claim in which the cells are exposed to anionic surfactant for not more than one minute, preferably not more than 30 seconds.
6. A method according to any preceding claim in which the thiol is dithiothreitol.
- 25 7. A method according to any preceding claim in which the anionic surfactant is an alkyl sulphate surfactant, preferably sodium dodecyl sulphate.
8. A process according to any preceding claim in which the borate salt is sodium ditetraborate.
- 30 9. A method according to any preceding claim in which the cells are treated with thiol at a concentration of from 25 mM to 1M, preferably from 75 mM to 300 mM, more preferably from 100 mM to 200 mM.
10. A method according to any preceding claim in which the  
35 cells are fixed with ethanol after treatment with thiol and anionic surfactant and optionally borate salt.

11. A method according to any preceding claim in which after treatment the cells are dried at a temperature of at least 30°C, preferably at least 40°C, more preferably at least 45°C.
- 5 12. A method according to claim 11 in which the sperm are dried for not more than 5 minutes, preferably not more than 2 minutes at the specified temperature.
13. A method according to any preceding claim in which the cell concentration is from  $10^7$  to  $10^{10}$  cells/ml, preferably  
10  $10^8$  to  $10^9$  cells/ml, more preferably  $1.5$  to  $3.5 \times 10^8$  cells/ml.
14. A method according to any preceding claim in which the ratio of cell concentration:thiol concentration is from 10,000,000 to 100,000 cells/ml:mM, preferably from 500,000  
15 to 5,000,000 cell/ml:mM.
15. A method according to any preceding claim in which the ratio of cell concentration:anionic surfactant concentration is from  $5 \times 10^7$  to  $5 \times 10^9$  cells/ml:% anionic surfactant (weight per volume).
- 20 16. A method according to claim 3 in which the ratio of cell concentration:borate salt concentration is from  $5 \times 10^7$  to  $5 \times 10^9$  cells/ml:% borate salt (weight per volume).
17. A method according to any preceding claim in which the cells are sperm cells, preferably livestock sperm.
- 25 18. A method according to claim 17 in which the sperm cells are ovine sperm, porcine sperm, equine sperm or bovine sperm, preferably bovine sperm.
19. A method of subjecting sperm to fluorescence in situ hybridisation (FISH) comprising decondensation of sperm DNA  
30 by means of a method according to any preceding claim.
20. A method according to claim 19 in which chromosome paints are used.
21. A method according to claim 19 or claim 20 in which the chromosome paints are produced by amplification of Yak  
35 X and Y chromosomes.
22. A method according to any of claims 19 to 21 in which after decondensation the sperm are treated with pepsin.

23. A kit for decondensation of cell DNA comprising (A) a solution of thiol and a concentration of at least 50mM and (B) a solution of anionic surfactant.
24. A kit according to claim 23 additionally comprising
- 5 (C) a solution of a borate salt.
25. A kit for decondensation of DNA comprising (A) thiol, (B) anionic surfactant and (C) instructions to treat the cells whose DNA is to be decondensed with the thiol and anionic surfactant such that the cells are exposed to thiol
- 10 for not more than 20 minutes.

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Fig.1a.

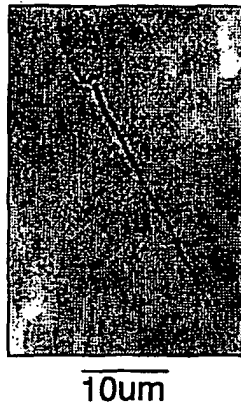


Fig.1b.

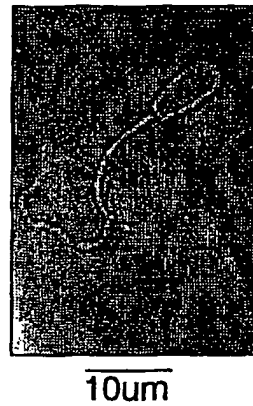


Fig.1c.

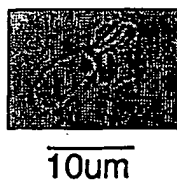


Fig.1d.

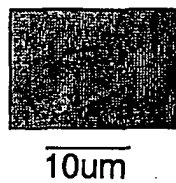


Fig.1e.

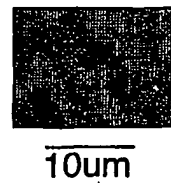


Fig.1f.

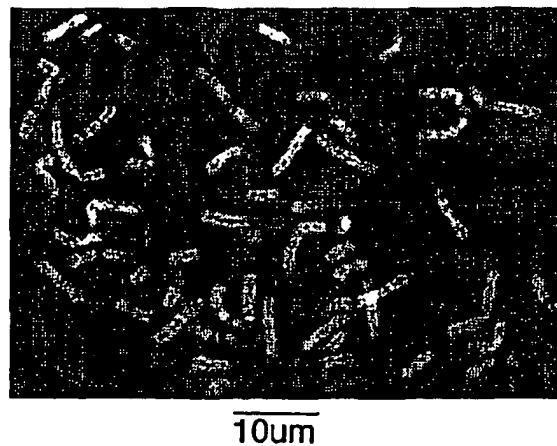
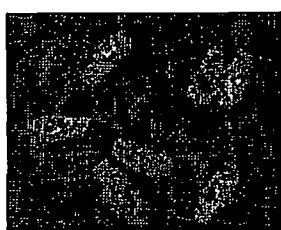


Fig.1g.



10um

Fig.1h.



10um

Fig.1i.



10um

Fig.1j.



10um

Fig.1k.



10um

Fig.1l.



10um